Direct Ras Inhibitors Identified from a Structurally Rigidified Bicyclic Peptide Library

Punit Upadhyaya, Ziqing Qian, Nurlaila A. A. Habir, and Dehua Pei

Supporting Information

Synthesis of Bicyclic Library. The bicyclic peptide library was synthesized on 2.0 g of TentaGel S NH_2 resin (90 μm, 0.26 mmol/g). All of the manipulations were performed at room temperature unless otherwise noted. The linker sequence (BBFM) was synthesized with 4 equiv of Fmoc-amino acids, using HBTU/HOBt/DIPEA as the coupling reagents. The coupling reaction was typically allowed to proceed for 1 h, and the beads were washed with DMF (3x) and DCM (3x). The Fmoc group was removed by treatment twice with 20% piperidine in DMF (5 + 15 min), and the beads were exhaustively washed with DMF (6x). To spatially segregate the beads into outer and inner layers, the resin was treated with 20% piperidine in DMF (5 + 15 min), washed with DMF and water, and soaked in water overnight. The resin was drained and suspended in a solution of Fmoc-OSu (0.26 mmol, 0.50 equiv) and diisopropylethylamine (1.2 mmol or 2.0 equiv) in 30 mL of 55:45 (v/v) DCM/diethyl ether. The mixture was incubated on a carousel shaker for 30 min at room temperature. The beads were washed with 55:45 DCM/diethyl ether (3x) and DMF (8x) to remove water from the beads and then treated with 5 equiv of Boc anhydride in DMF. Next, 2 equiv of 4-hydroxymethylbenzoic acid was added to the resin with HBTU/HOBt/DIEA (2:2:4 equiv) after the removal of Fmoc group. Fmoc-β-Ala-OH (5 equiv.) was coupled to the Hmb linker by using DIC/DMAP (5.5:0.1 equiv.), and the coupling was repeated twice to drive the reaction to completion. Then, Fmoc-L-Pra-OH, two Fmoc-β-Ala-OH, and Fmoc-L-Dap(Alloc)-OH were sequentially coupled by standard Fmoc/HBTU chemistry. The Boc protecting group on the encoding sequence was removed by TFA/water/triisopropylsilane (95:2.5:2.5), followed by the coupling of Fmoc-Arg(Pbf)-OH. The random region was synthesized by the split-and-pool method using 5 equivalents of Fmoc-amino acids and HATU as the coupling agent. The coupling reaction was repeated once to ensure complete reaction at each step. To differentiate isobaric amino acids during PED-MS sequencing, 4% (mol/mol) of CD_3CO_2D was added to the coupling reactions of D-Ala, D-Leu, D-Lys, and Orn, while 4% $CH_3CD_2CO_2D$ was added to the Nle reactions. Fmoc-Lys(Mmt)-OH was placed in the middle of the random positions using HATU/DIPEA (4 equiv, 8 equiv) to facilitate the formation of bicyclic compounds. After the synthesis of random positions, the Alloc group on the C-terminal Dap was removed with a solution containing tetrakis(triphenylphosphine)palladium (0.25 equiv) and phenylsilane (5 equiv) in anhydrous DCM for 15 min (3x). The beads were sequentially washed with 0.5% diisopropylethylamine in DMF, 0.5% sodium dimethyldithiocarbamate hydrate in DMF, DMF (3x), DCM (3x), and DMF (3x). The resulting free amine was coupled to diallyl protected trimesic acid using HATU/DIPEA (5 equiv, 10 equiv) for 2 h. The allyl groups on trimesic acid scaffold were removed using the same procedure as described for Alloc deprotection. The Mmt group on lysine(Mmt) was removed using 2% TFA /5% TIPS in DCM for 40 min. The N-terminal Fmoc group was then removed with 20% piperidine. The beads were washed with DMF (6x), 1 M HOBt in DMF (3x), DMF (3x), and DCM (3x). For peptide cyclization, a solution of PyBOP/HOBt/NMM (5, 5, 10 equiv, respectively) in DMF was mixed with the resin and the mixture was incubated on a carousel shaker for 3 h. The resin was washed with DMF (3x) and DCM (3x) and dried under vacuum for >1 h. Side chain deprotection was carried out with reagent K (7.5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol, 1% anisole, and 1% triisopropylsilane in TFA) for 1 h. The resin was washed with TFA and DCM and dried under vacuum before storage at -20 °C.

Cell Culture. A549 cells were maintained in medium consisting of DMEM, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. H358 cells were maintained in medium consisting of

RPMI-1640, 10% FBS and 1% penicillin/streptomycin. Cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

Confocal Microscopy. To detect peptide internalization, 1 mL of A549 cell suspension (5 x 10^4 cells) was seeded in a 35 mm glass-bottomed microwell dish (MatTek) and cultured overnight. Cells were gently washed with DPBS twice and treated with FITC labeled peptides (5 μM) and rhodamine B-dextran ($Dextran^{Rho}$, 0.5 mg mL^{-1}) in phenol-red free DMEM containing 1% serum for 37 °C for 1 h in the presence of 5% CO_2. After removal of the medium, the cells were gently washed with DPBS twice and incubated with 5 μM DRAQ5 in DPBS for 10 min. The cells were again washed with DPBS twice and imaged on a Visitech Infinity 3 Hawk 2D-array live cell imaging confocal microscope.

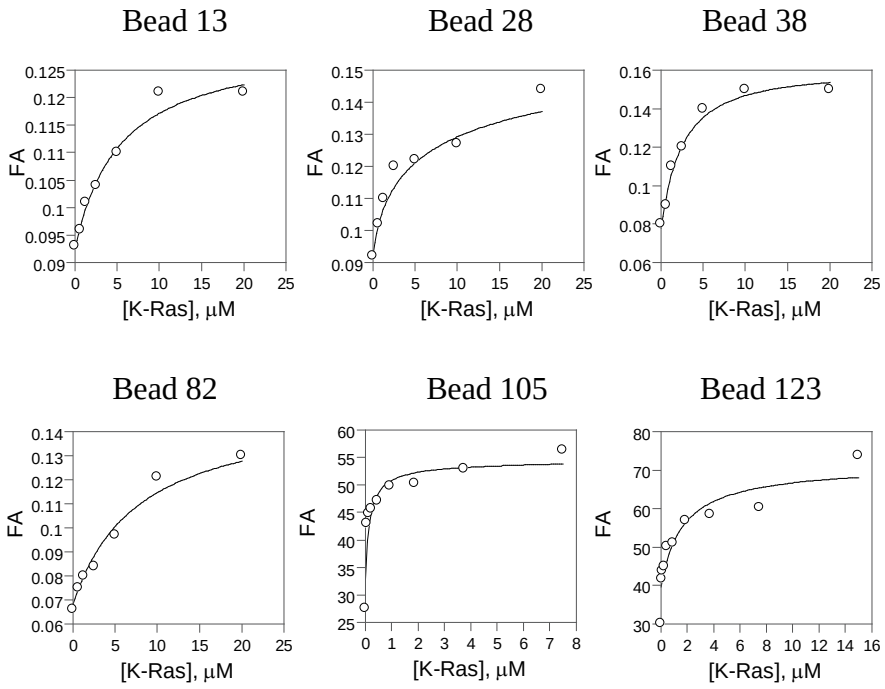
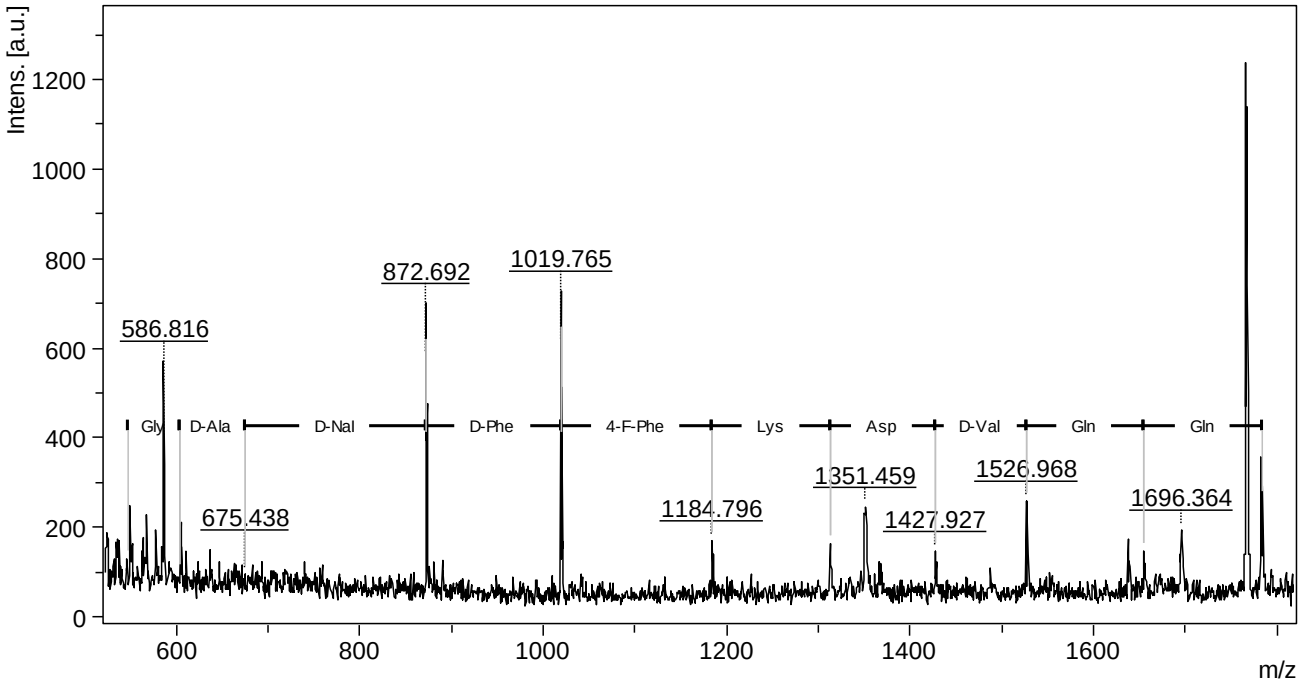


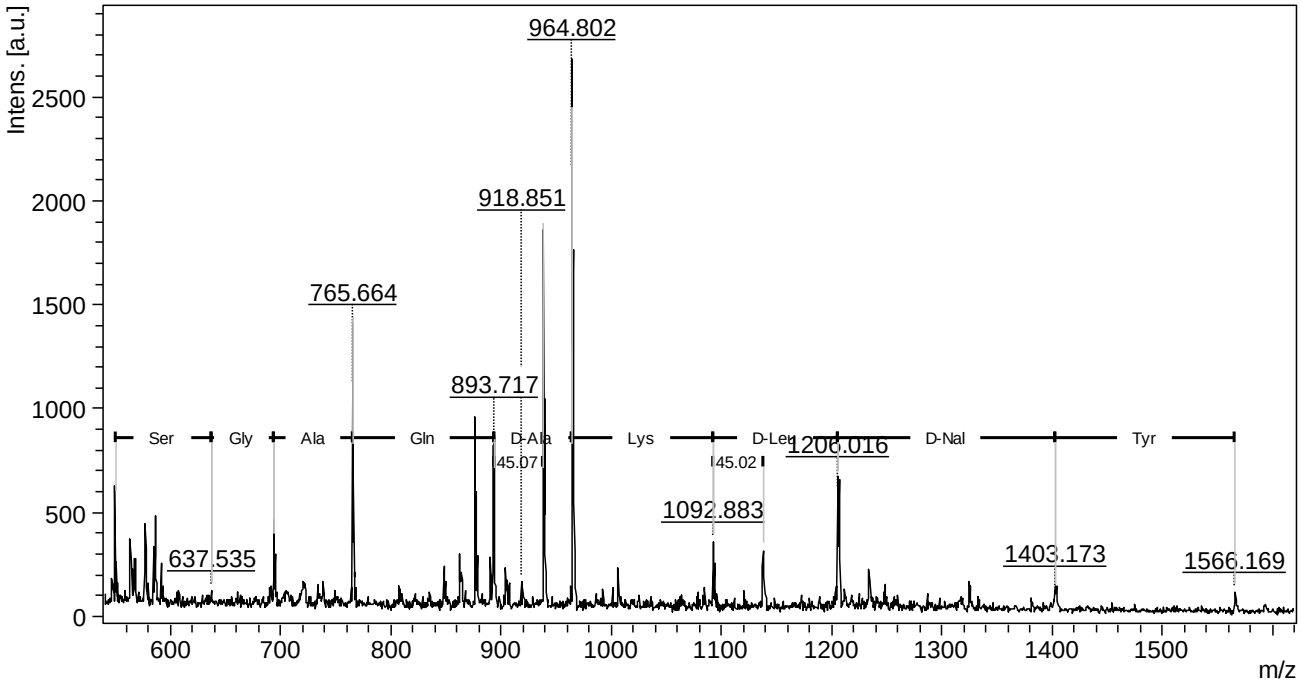
Figure S1. FA analysis of K-Ras-G12V binding by TMR-labeled bicyclic peptides released from single beads. Each TMR-labeled bicyclic peptide (final concentration ~50 nM) was incubated with varying concentrations of K-Ras and the FA values were plotted against the K-Ras concentration. Curve fitting (as described in main text) gave the K_D values.

Figure S2. PED-MS Sequencing of hit peptides. Positive beads after the solution-phase screening (4th round) were subjected to 11 cycles of PED, and the peptides were released from each bead by CNBr, and analyzed by MALDI-TOF MS. The M+45 and M+58 peaks were used to differentiate isobaric amino acids (e.g., Leu and Ile). M* = homoserine lactone formed after CNBr cleavage.

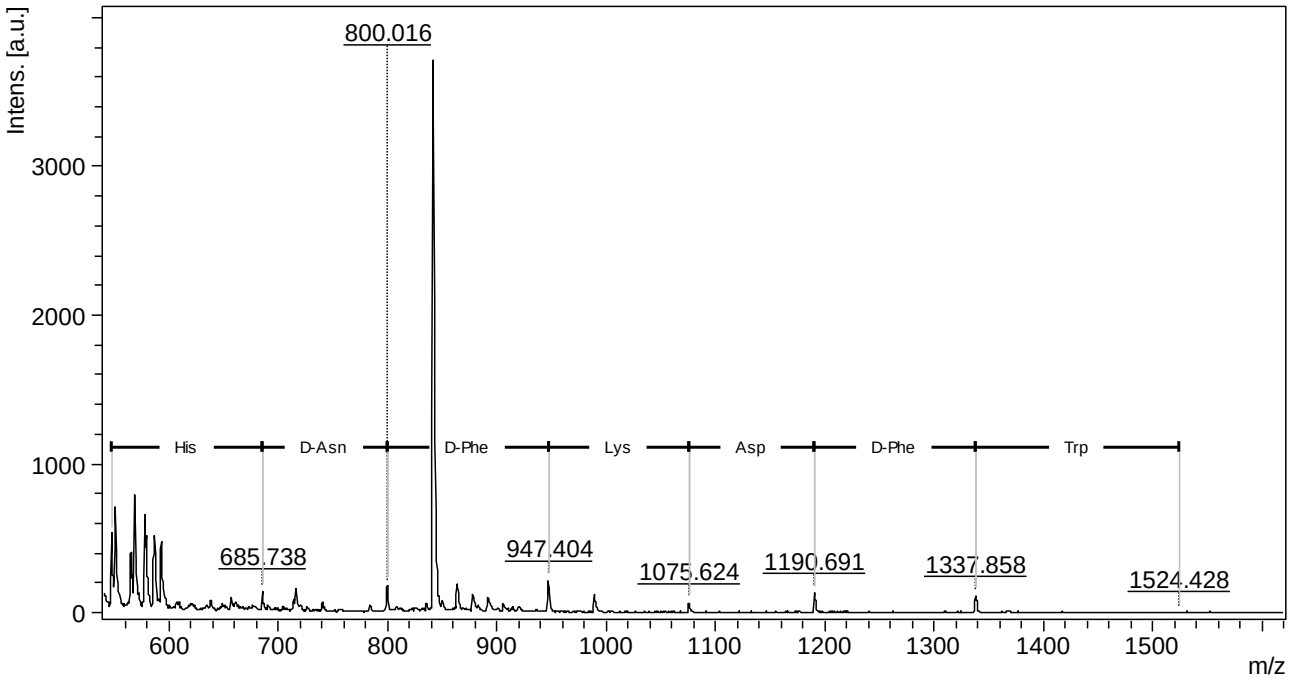
Bead 13: Gln-Gln-D-Val-Asp-Lys-4-F-Phe-D-Phe-D-Nal-D-Ala-Gly-RBBFM*



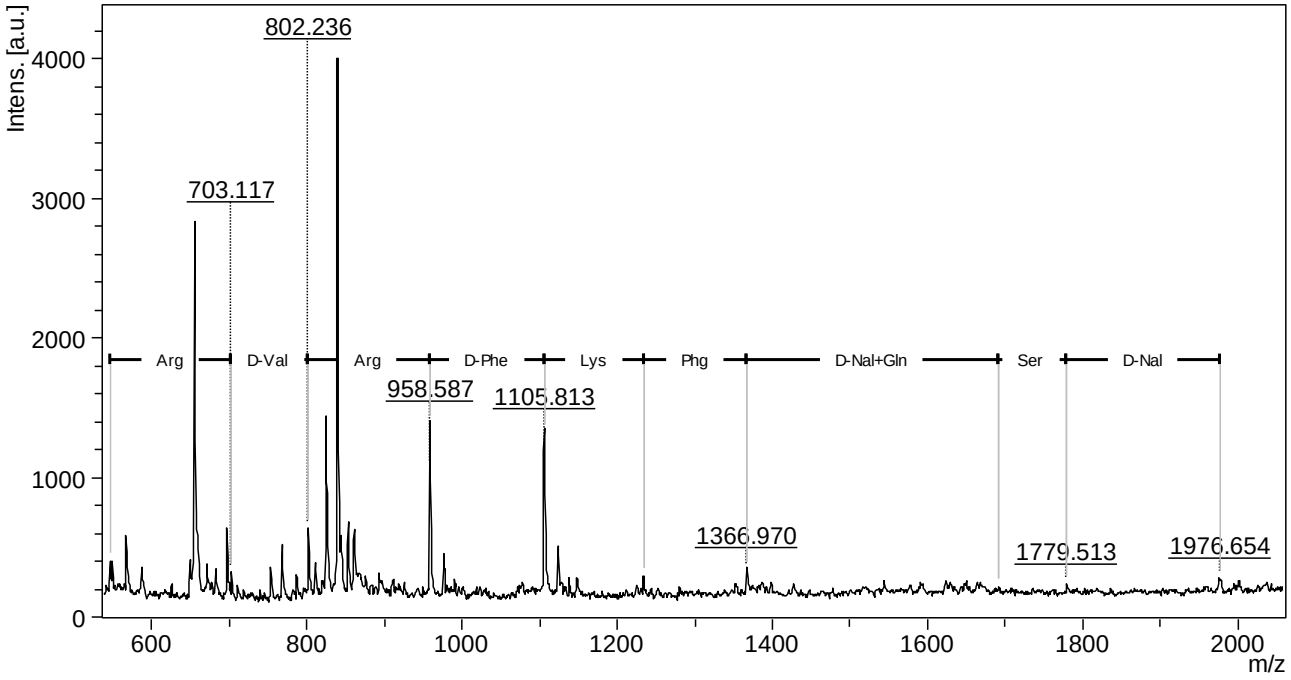
Bead 28: Tyr-D-Nal-D-Leu-Lys-D-Ala-Gln-Ala-Gly-Ser-RBBFM*



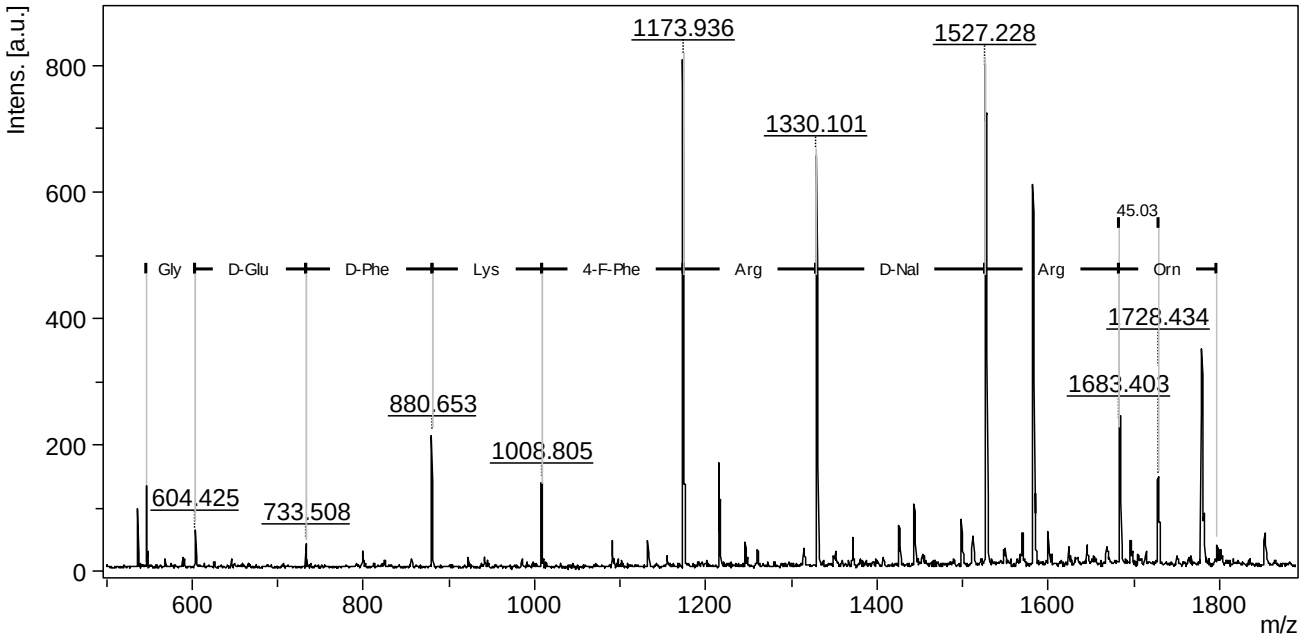
Bead 38: Trp-D-Phe-Asp-Lys-D-Phe-D-Asn-His-RBBFM*

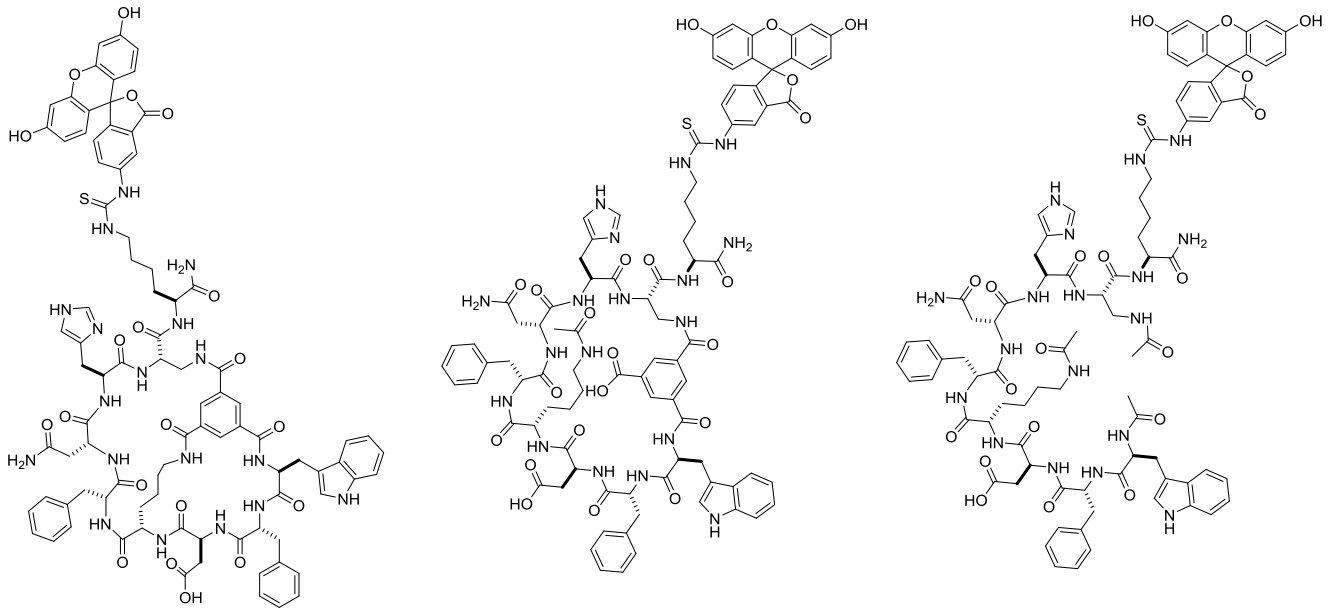


Bead 82: D-Nal-Ser-D-Nal-Gln-Phg-Lys-D-Phe-Arg-D-Val-Arg-RBBFM*



Bead 105: Orn-Arg-D-Nal-Arg-4-F-Phe-Lys-D-Phe-D-Glu-Gly-RBBFM*





FITC-cyclorasin B2 FITC-monocyclic analog FITC-linear analog

Figure S3. Structures of FITC-labeled cyclorasin B2 and its monocyclic and linear counterparts.

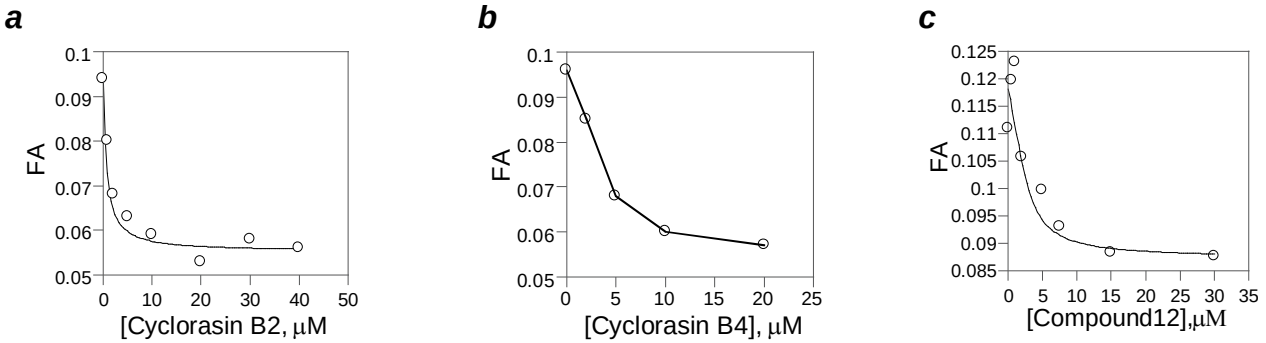


Figure S4. Competition for binding to K-Ras among the Ras ligands as analyzed by FA. (***a***) Effect of unlabeled cyclorasin B2 on binding of FITC-cyclorasin B2 to K-Ras. (***b***) Effect of unlabeled cyclorasin B4 on binding of FITC-cyclorasin B4 to K-Ras. (***c***) Inhibition of FITC-cyclorasin binding to K-ras by compound 12.

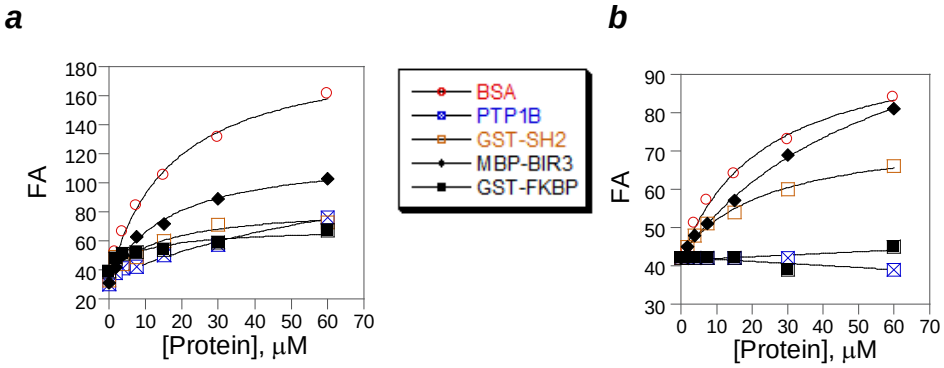


Figure S5. FA analysis of the binding of FITC-labeled cyclorasin B2 (***a***) and B3 (***b***) to five arbitrarily selected control proteins.

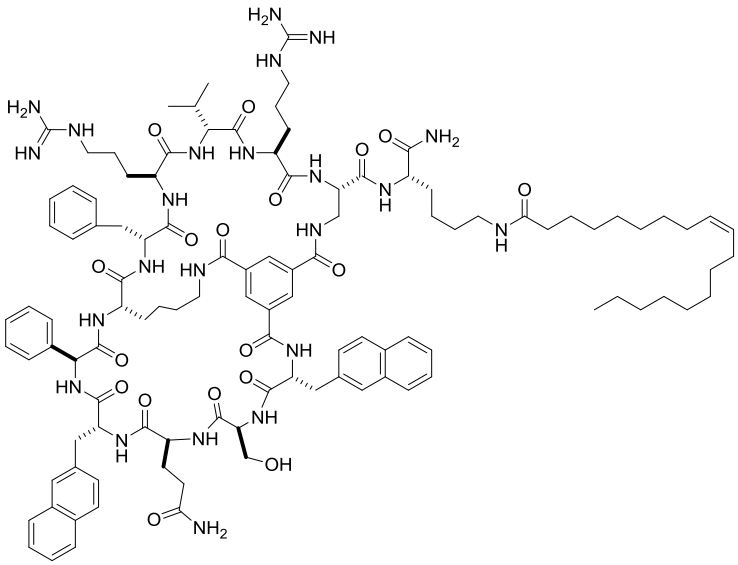


Figure S6. Structure of cyclorasin B3 conjugated to an oleic acid.